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Reduction of *Brochothrix thermosphacta* on beef surfaces following immobilization of nisin in calcium alginate gels

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C.N. CUTTER AND G.R. SIRAGUSA. 1996. Lean and adipose beef carcass tissues inoculated with *Brochothrix thermosphacta* (*BT*) (approx. 4·50 $\log_{10}$ cfu cm^{-2}) were left untreated (U) or treated with 100 μg ml^{-1} nisin (N), calcium alginate (A) or 100 μg ml^{-1} nisin immobilized in a calcium alginate gel (AN). Tissue samples were refrigerated after treatments and bacterial populations and nisin activity were determined at 0, 1, 2 and 7 d. U, A and N treatments of lean and adipose tissues did not suppress bacterial growth (>6 $\log_{10}$ cfu cm^{-2} by day 7) while treatments of lean and adipose tissues with AN suppressed bacteria ($>2·42$ $\log_{10}$ cfu cm^{-2} by day 7). Bacteriocin titres from both tissues were higher in AN *vs* N samples after the 7 d incubation. This study demonstrates that immobilization of nisin in a gel may be a more effective delivery system of a bacteriocin to the carcass surface than direct application.

INTRODUCTION

Encasing foods in protective wraps, casings, coatings or films is a well documented form of preservation. The United States beef industry currently distributes 93% of its product in either a vacuum or modified atmosphere package (personal communication, Charles Jolley, Cryovac, Duncan, SC) for prolonging the shelf-life of its products. Cutter and Siragusa (1996) have demonstrated the efficacy of nisin spray washes and vacuum packaging for suppressing the growth of Gram-positive bacteria on beef over a 4-week period. While Gram-positive bacteria were significantly reduced by simple nisin/vacuum packaging combinations, other methods of nisin application with sustainable reductions warrant investigation.

Meyer *et al.* (1959) demonstrated that antibiotics and antifungal compounds could be added to a carageenan film to reduce bacteria by 2 $\log_{10}$ on poultry. More recently, Siragusa and Dickson (1992, 1993) have demonstrated that organic acids were more efficacious for reducing levels of *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli* O157 : H7 when immobilized in calcium alginate and applied to beef carcass tissue than when these compounds were applied alone. Baron (1993) demonstrated that potassium sorbate and lactic acid could be incorporated into an edible cornstarch film to inhibit *Salm. typhimurium* and *E. coli* O157 : H7 on poultry. To our knowledge, there is no information pertaining to the incorporation of bacteriocins into edible films or gels. The following study was performed to determine whether immobilization of nisin by a calcium alginate gel was effective for inhibiting *Brochothrix thermosphacta*, a major spoilage organism of fresh, vacuum-packaged red meat.

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Names are necessary to report factually on available data ; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

MATERIALS AND METHODS

Bacterial cultures and inoculation of beef

Brochothrix thermosphacta ATCC 11509 (*BT*) was maintained in 75% glycerol at −20°C. *BT* was propagated in trypticase soy broth supplemented with 0·5% yeast extract (TSBYE; Troy Biologicals, Troy, MI) at 25°C for 18 h.

Lean and adipose tissues from the outer surfaces of post rigor (24 h post mortem) beef carcasses were obtained from the Roman L. Hruska US Meat Animal Research Center (RLHUSMARC) abattoir, vacuum packaged, and stored at −20°C. Prior to experiments, tissue was thawed to 25°C, trimmed to approximately 8 cm × 8 cm × 0·5 cm, and surface-sterilized by u.v. light (Cutter and Siragusa 1994). Sterility

was monitored by individually sampling six pieces of lean or adipose tissue at day 0 using the enumeration procedures described below. Overnight cultures of *BT* were diluted 1 : 100 in sterile physiological saline (pH 7·0) to obtain a viable cell population of approximately 8 $\log_{10}$ cfu ml^{-1}. The individual pieces of lean and adipose tissues were placed onto a sterile tray, the inoculum was brushed onto the tissue with a sterile 2-inch wide paint brush, and the tissue pieces were incubated for 15 min, 25°C (Dorsa *et al.* 1996) prior to applying treatments. Bacterial populations of approximately 4·50 $\log_{10}$ cfu cm^{-2} were obtained using this procedure.

Bacteriocin preparation and activity assays

Purified nisin (Ambicin™, Applied Microbiology, New York, NY) was solubilized in distilled water, filter sterilized (0·2 μm Acrodisc™, Gelman Sciences, Ann Arbor, MI), added to sterile distilled water for a final stock concentration of 1 mg ml^{-1} (pH 6·0) and stored at −20°C. Prior to experiments, the stock solution was thawed to 25°C and diluted 1 : 10 in sterile distilled water or sterile alginate solution for a final nisin concentration of 100 μg ml^{-1} used throughout this study.

Spot assays were performed on lawns of *BT* to monitor bacteriocin activity in solutions containing nisin, as well as stomachates, and tissue samples as previously described (Siragusa 1992). Residual nisin activity on lean and adipose tissue samples was determined by a modified acid-boil procedure (Fang and Lin 1994) as follows: 3·5 g of tissue were added to a sterile tube containing 35 ml of 0·02 mol l^{-1} hydrochloric acid (pH 2·0). The sample was placed into a boiling water bath for 5 min and centrifuged for 10 min at 3500 rev min^{-1}, 5°C. Ten μl of the supernatant fluid were used in spot assays (Siragusa 1992). Plates were incubated overnight at 26°C. All samples exhibiting activity during initial nisin assays were stored at −20°C, thawed to 25°C, titred to extinction on lawns of *BT*, and the reciprocal of the highest dilution exhibiting activity was recorded (Siragusa 1992).

Treatments

One per cent (w/v) high viscosity sodium alginate (pH 7·0; Sigma Chemical Co., St Louis, MO) and 90 mmol l^{-1} calcium chloride (pH 7·0; $CaCl_2$, Sigma) were prepared in distilled water and sterilized. Lean and adipose tissues inoculated with *BT* were separated into four batches and treated as follows: untreated (U); treated with 10 ml of alginate solution and cross linked with 10 ml of $CaCl_2$ (A); treated with 10 ml of 100 μg ml^{-1} nisin (N); treated with 10 ml of alginate solution containing 100 μg ml^{-1} nisin and cross linked with 10 ml of $CaCl_2$ (AN). All treatments were performed at 25°C within 30 s using a sterile 7·5 cm × 7·5 cm template to hold the liquid on top of the tissue in order to maintain contact with the tissue surface. All tissues were aseptically stored at 4°C until sampled at days 0, 1, 2 or 7.

Bacterial enumeration

At 0, 1, 2 or 7 d of refrigeration, a 25 cm^2 piece was aseptically excised from the untreated or treated tissues, stomached for 2 min (Stomacher 400, Tekmar, Inc., Cincinnati, OH) in a Sterefil™ Stomacher bag (Spiral Biotech, Bethesda, MD) with 25 ml of buffered peptone water (BPW, pH 7·0; BBL, Cockeysville, MD) containing 0·1% Tween 20 (Fisher, St Louis, MO). Each stomachate was serially diluted in BPW, and spiral plated (Model D Spiral Plater; Spiral Biotech, Bethesda, MD) in duplicate on trypticase soy agar (TSA; Difco, Detroit, MI) supplemented with 0·5% yeast extract (Difco; TSAYE). Plates were enumerated with a CASBA IV image analyzer (Spiral Biotech) after incubation for 36 h at 25°C. Since residual nisin did not interfere with plating procedures in a previous study (Cutter and Siragusa 1994), enzymatic inactivation of nisin was not included in the experimental design of this study.

After excising the 25 cm^2 section for bacterial enumeration, remaining pieces of untreated and spray-treated tissues were stored in sterile Whirl-Pak™ bags (Nasco, Fort Atkinson, WI) and used to assess residual nisin activity (Fang and Lin 1994).

Calculations and statistical analyses

The experiment was a 2 (tissue types) × 4 (treatments) × 4 (d) factorially arranged, completely randomized design. After enumeration, bacterial populations were converted to $\log_{10}$ cfu cm^{-2}. Least squared means (LSM) of bacterial populations ($\log_{10}$ cfu cm^{-2}) were calculated from six experimental replications. Analysis of variance was performed using the General Linear Models procedure of SAS (SAS Institute, ver. 6.06.01, 1989, SAS Inst., Inc., Cary, NC, 1982). Inoculum counts were used as a covariant to normalize data between treatment replications. Statistical significance was defined as $P \leqslant 0{\cdot}05$, unless otherwise noted.

Nisin titres were converted to the reciprocal of the highest dilution exhibiting activity and these values were used for analyses. LSM of nisin titres from stomachates or acid-boil samples were calculated from six experimental replications. Analysis of variance (ANOVA) was performed using the General Linear Models (GLM) procedure of SAS. The probability level for population or nisin titre data was $P \leqslant 0{\cdot}05$, unless otherwise noted.

RESULTS AND DISCUSSION

ANOVA of population data demonstrated that the effects of tissue, treatment and day were significant ($P < 0{\cdot}0001$) as

were the 2-way interactions of tissue by day and treatment by day. Populations of *BT* on lean and adipose beef tissue left untreated (U) and following treatments with alginate (A), nisin (N; 100 μg ml^{-1}) and alginate solutions containing nisin (AN; 100 ug ml^{-1}) are presented in Figs 1 and 2, respectively.

Initial bacterial populations of lean and adipose tissues treated with A were greater than U. However, bacterial populations from A-treated lean and adipose tissues were not statistically different from untreated tissues. Ultimately, U- and A-treated populations grew to greater than 6 $\log_{10}$ after 7 d under refrigerated conditions. Initially, N treatments of inoculated lean and adipose tissue resulted in a 2·84 and a 2·91 $\log_{10}$ reduction of *BT*, respectively. Slight inhibition of *BT* was observed after 7 d following N treatment, indicating some residual nisin activity. These data are similar to a previous study in which we demonstrated that nisin sprays of beef tissue resulted in initial reductions of 3 $\log_{10}$ of *BT* on lean tissue and 2 $\log_{10}$ on adipose with sustained reductions of approximately 2 $\log_{10}$ on lean and 3·6 $\log_{10}$ on adipose tissues after 24 under refrigeration (Cutter and Siragusa 1994).

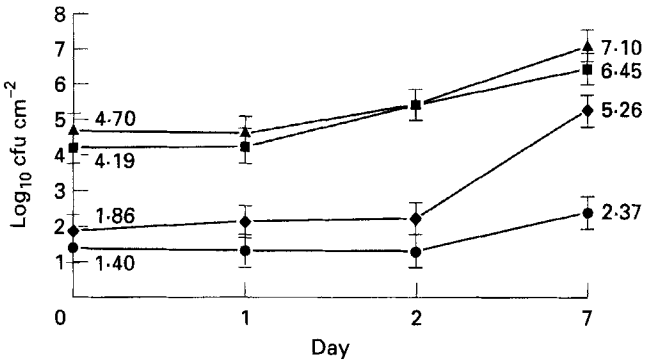

Fig. 1 Populations of *Brochothrix thermosphacta* on lean beef tissue left untreated (U, ■) or following treatments with alginate (A, ▲), nisin (N, ◆; 100 μg ml^{-1}) and alginate containing nisin (AN, ●; 100 μg ml^{-1}) and refrigerated storage up to 7 d

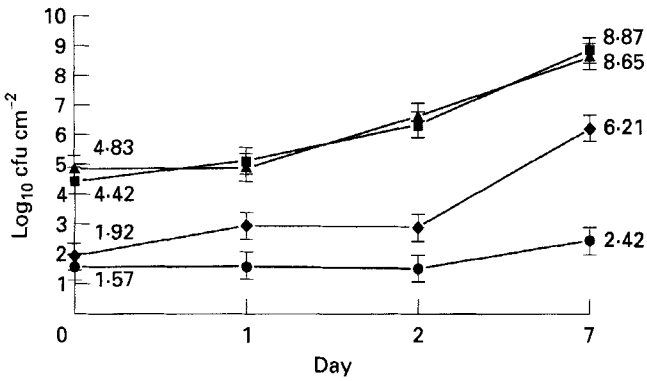

Fig. 2 Populations of *Brochothrix thermosphacta* on adipose beef tissue left untreated (U, ■) or following treatments with alginate (A, ▲), nisin (N, ◆; 100 μg ml^{-1}) and alginate containing nisin (AN, ●; 100 μg ml^{-1}) and refrigerated storage up to 7 d

Treatments with AN resulted in > 3 $\log_{10}$ reductions at day 0 with sustained reductions (> 4 $\log_{10}$) at 7 d of refrigerated storage, regardless of tissue type. Siragusa and Dickson (1992, 1993) have demonstrated that bacterial reductions were greater following immobilization of antimicrobial compounds (e.g. organic acids) in calcium alginate gels than when the compounds were applied alone (Siragusa and Dickson 1992, 1993). The data from the present study demonstrate that immobilization of nisin in a calcium alginate gel is more effective for reducing bacterial populations than applying nisin in a liquid form.

Nisin activity was not detected in stomachates from any of the untreated or alginate-treated lean and adipose beef tissue. Nisin titres taken from stomachates and tissue subjected to the acid-boil procedure are depicted in Figs 3 and 4. ANOVA of titre data taken from both nisin assays performed on N- and AN-treated tissue demonstrated that treatment was a significant effect (data not presented). Overall, the highest titres of nisin were observed in stomachate samples rather than samples subjected to the acid-boil procedure. Regardless of assay method employed, nisin activity was consistently greater in samples taken from tissues subjected to alginate solutions containing nisin (AN) than tissues treated with nisin (N) alone. When bacteriocins are applied directly to a meat surface in a liquid form, there are several possibilities for diminished activity, including degradation of the protein by endogenous proteases associated with the surface of red meat, adsorption of nisin onto meat proteins or lipid particles, or assays that are not sensitive enough to detect the bacteriocin in samples (Bell and DeLacy 1986; Ray 1992; Nettles and Barefoot 1993). In this study and another previous study,

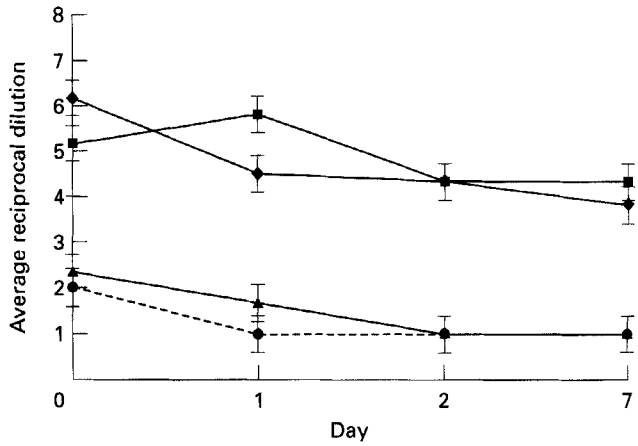

Fig. 3 Nisin titres taken from stomachates of lean (L) and adipose (A) beef tissue treated with nisin (N) and alginate containing nisin (AN). ◆, AN-F; ■, AN-L; ▲, N-F; ●, N-L

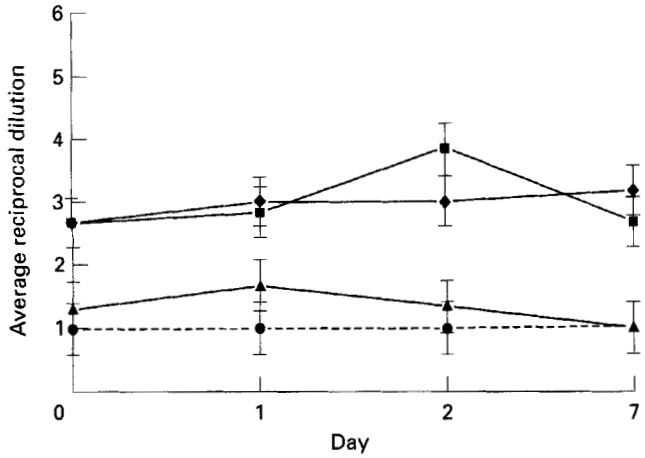

Fig. 4 Nisin titres taken from lean (L) and adipose (A) beef tissue treated with nisin (N) and alginate containing nisin (AN) and subjected to a modified acid-boil procedure. ◆, AN-F; ■, AN-L; ▲, N-F; ●, N-L

bacteriocin activity from N-treated tissues was significantly diminished within 24 h after application (Cutter and Siragusa 1994). In both instances, bacterial populations remained suppressed, as compared to untreated controls, indicating that nisin was still active for the duration of the experiment. The titre data obtained from stomachates in these experiments suggest that immobilization of nisin in a calcium alginate gel on a beef surface results in sustained greater nisin activity for a longer length of time.

To our knowledge, this study represents the first report in which a bacteriocin has been immobilized in a calcium alginate gel. In summary, our results indicate that the immobilization of nisin not only results in greater reductions of bacterial populations on a beef surface, but also results in sustained bacteriocin activity up to 7 d under refrigerated conditions.

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